THE ACTION OF ACETYLCHOLINE, ATROPINE AND PHYSOSTIGMINE ON THE INTESTINE OF DAPHNIA MAGNA

VASIL OBRESHKOVE

(From Bard College, Columbia University)

Introduction

The effects of acetylcholine when administered to animals under experimental conditions have recently afforded valuable information which suggests the possibility that acetylcholine is acting as a chemical transmitter of nervous impulses from nerve endings to certain organs of the body. In view of the excitatory action of acetylcholine which has been demonstrated on the heart of crustaceans (Welsh, 1939 *a* and *b*) and the influence of this substance on the autotomy of certain members of this group of animals (Welsh and Haskin, 1939), it appears worth while to investigate the action of this drug on the intestine of a cladoceran, a problem heretofore unexplored. Although there is nothing in Cladocera which corresponds morphologically to the autonomic nervous system in vertebrates, the intestine of these animals is subject to accelatory and inhibitory nervous influences. If the intestine of *Daphnia magna*, for example, is touched with a very fine glass needle at the bend of the digestive tube where the intestine enters the stomach, the heart immediately stops beating and the posterior region of the intestine commences to exhibit powerful intestinal contractions. After a certain period, depending on the strength of the mechanical stimulus applied, the heart renews its activity and the intestine reestablishes its normal muscular contractions. If acetylcholine is involved in the transmission of nervous impulses to this organ, it should be possible to obtain some evidence of the action of this substance and other substances with which it has been said to be associated, when they are administered to this animal.

Methods

Daphnia magna young, when in their second instar, were used exclusively for the experimental work. The animals at this stage measure about 1 mm. in length, they are more transparent than the adult individuals and hence the changes produced in the course of the experimentation can be easily observed under the microscope. The mothers from

which the young were obtained for the experiments were reared at 25° C. in bottles containing the standard amount of the culture medium (Banta, 1921). The daily examination of the animals and the other methods employed in rearing the organisms in the laboratory (Obreshkove, 1930) enabled us at all times in the course of the experimental work to secure animals which were of the same age. A careful selection of the animals was necessary, because of the endeavor which was made to measure the period which elapsed between the addition of the particular chemical substances under investigation and the characteristic changes which they produced. The chemical substances employed were acetylcholine chloride, physostigmine (eserine) and atropine. Care was taken to use freshly diluted chemicals. The acetylcholine was adjusted to pH 5.7.

The animals were subjected to experimentation separately. A single individual was transferred to a micro culture slide with polished spherical concavity 18 mm. in diameter and approximately 3 mm. deep. The culture medium surrounding the animal was removed and immediately after this there was added the chemical substance whose action on the animal was to be studied. The amount of solution employed in each depression slide was kept the same and in each case it was just sufficient to cover the animal without permitting it to carry on extensive locomotive movements. This procedure enabled us to make continuous observations on a single individual under the microscope. The animal is seen at all times to ingest solid particles and fluid with which it comes in contact in the depression slide. With each opening of the mouth, a quick and powerful peristaltic wave of the esophagus forces the ingested material into the stomach, a process which can be easily observed under the microscope. Normally about 40 such peristaltic waves occur each minute. It is suggested, therefore, that the drugs employed in this work were administered orally.

Experimental Results

The intestine of untreated animals usually exhibits movements which are more or less rhythmic in nature. There is a gentle surging back and forth of the nutritive material and only when the animal is in the act of evacuating the contents of the intestine is one able to observe peristaltic and antiperistaltic waves in the musculature of the organ itself. At such times the forward peristalsis becomes more noticeable than the reverse peristalsis, the anus opens and the animal excretes only a small portion of the intestinal contents. This act is repeated at irregular intervals which vary from 30 seconds to more than 1 minute in some cases. At no

time, however, is the intestine entirely empty, for in the depression slide the animal is continuously reëngulfing the materials which it has evacuated.

The Action of Acetylcholine

When a *Daphnia magna* young is treated with acetylcholine, a very distinct change occurs in the intestine. The muscular peristaltic and antiperistaltic contractions of the organ become extremely violent and when stronger solutions are employed the entire contents of the intestine are emptied in a little more than a minute. The time which elapses between the application of the drug and the appearance of the first vigorous muscular contraction varies very definitely with the concentration of the drug employed. From an inspection of Table I it is seen that with acetylcholine 1×10^{-2} this occurs on the average in less than 20 seconds and with acetylcholine 1×10^{-3} this period is increased to 27.4 seconds. There is not a gradual development in the establishment of the violent intestinal activity. When the drug becomes effective, it exhibits its effectiveness to the fullest extent with an abrupt initial powerful contractile wave of considerable amplitude. After treatment with acetylcholine 1×10^{-3} and subsequent transference to water, vigorous forward and reverse peristalsis will continue in some cases for as long as 20 or 30 minutes. Acetylcholine 1×10^{-2} with lapse of time produces high intestinal tone and contracture.

When *Daphnia magna* young are treated with acetylcholine 1×10^{-4}, the time which elapses between the addition of the drug and the first appearance of the characteristic effect produced is on the average 10.7 minutes for the group of experiments presented here (Table I, column 3). With further dilution of the drug this period becomes longer. With acetylcholine 1×10^{-5} the time varies from 50 to 137 minutes (Table I, column 4), showing a definite and considerable increase over the time of reaction obtained with the higher concentrations of the drug.

The Action of Atropine

Atropine was found to antagonize the action of acetylcholine. When *Daphnia magna* are treated with acetylcholine until the characteristic powerful action of the intestine is established and then the solution is replaced by atropine, the effects of acetylcholine are quickly abolished. The powerful contractions, which would otherwise persist for many minutes, not only disappear but in many individuals after the atropine has become fully effective there is no longer any evidence of intestinal muscular contractions. Atropine 10^{-2} abolishes the effect of acetylcholine of

the same concentration in less than 20 seconds, but atropine was found to be effective even in dilutions of 1×10^{-9}. The range of effectiveness beyond this concentration of the drug was not investigated. The results obtained with acetylcholine 1×10^{-3} and atropine 1×10^{-5} are shown in Table II. The rapidity with which acetylcholine 1×10^{-3} produced its characteristic action on the intestine is shown here to be no different from that previously recorded in this paper (Table I). Atropine 1×10^{-5}, on the other hand, repeatedly abolished the effects of acetylcholine within 20 to 52 seconds. Table II also shows that following the abolishing of the powerful intestinal contractions by atropine, a stronger solution of

TABLE I

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine of various concentrations. The time of action is expressed in seconds or minutes and represents the period elapsing from the addition of the drug to the appearance of the characteristic effect.

Acetylcholine 1×10^{-2}	Acetylcholine 1×10^{-3}	Acetylcholine 1×10^{-4}	Acetylcholine 1×10^{-7}
seconds	*seconds*	*minutes*	*minutes*
20	25	10.8	119
20	35	8.2	137
22	40	8.2	123
19	30	14.4	125
22	30	12.3	74
15	20	9.3	113
16	22	11.7	110
17	25	12.2	124
19	20	9.3	50
24	27	10.8	69
Average 19.4	27.4	10.7	104.4

acetylcholine (1×10^{-2}) reëstablished the previous effect of acetylcholine, the average time for this being 21.8 seconds—a reaction time characteristic for this concentration of the drug (compare with Table 1).

Acetylcholine and atropine of the dilutions employed in this work produced no lethal effect on the animals. Likewise atropine, when it was repeatedly administered to the same individual after treatments with acetylcholine, had no paralytic effect on the musculature of the intestine. To test this, a single individual was subjected to experimentation in the following way. The animal was treated with acetylcholine 1×10^{2}. Immediately after the appearance of strong intestinal contractions, the drug was removed and replaced with atropine 1×10^{-5}. After the abolishing of the muscular contractions, the animal was again treated with acetylcholine and then atropine of the same dilutions as pre-

TABLE II

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine; the time of abolishing the acetylcholine effect by atropine; and the time of reestablishment of strong contractions by acetylcholine following atropine.

Acetylcholine 1×10^{-3}	Atropine 1×10^{-5}	Acetylcholine 1×10^{-2}
Time of action	Time of abolishing acetylcholine effect	Time of action of acetylcholine 10^{-2} after atropine
seconds	*seconds*	*seconds*
29	30	20
26	30	25
25	40	22
26	52	18
34	22	35
38	36	32
22	38	20
38	20	18
24	38	20
32	34	22
28	35	18
34	42	18
34	38	20
43	42	20
22	40	19
Average 30.3	35.8	21.8

TABLE III

The effects of repeated treatment of a single *Daphnia magna* with acetylcholine and atropine at regular intervals of a few seconds.

Acetylcholine 1×10^{-2}	Atropine 1×10^{-5}
Time of action	Time of abolishing acetylcholine effect
seconds	*seconds*
20	90
20	110
18	80
14	80
16	95
13	70
14	110
14	80
28	52
18	85
Average 17.7	85.2

viously employed. This procedure was repeated on the same individual for ten times and the results which were obtained are shown on Table III. It is evident from an inspection of the table that the drugs continued after each application to produce their characteristic effects. Atropine 1×10^{-5} under the conditions employed in this set of experiments required on the average 85.2 seconds to produce its characteristic effect, in comparison with 35.8 seconds (Table II), which was required for the drug of this dilution to block the effect of acetylcholine 1×10^{-3}. This difference in the reactivity is apparently due to the fact that the treatment with atropine in this particular set of experiments was preceded by a stronger solution of acetylcholine (1×10^{-2}) than heretofore employed in studying the antagonistic effect of atropine.

TABLE IV

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine (1×10^{-7}) following the administration of physostigmine (1×10^{-4} for 2 minutes) and the action of physostigmine 1×10^{-4} when administered alone.

Time of action of acetylcholine 1×10^{-7} after eserinization	Time of action of physostigmine 1×10^{-4}
seconds	*minutes*
45	9.9
47	10.0
52	10.2
58	10.0
41	9.7
55	10.3
70	9.4
37	10.1
42	9.7
30	9.6
51	9.9
41	10.4
97	10.7
52	9.5
34	9.5
Average 50.1	9.9

THE ACTION OF PHYSOSTIGMINE

Physostigmine (eserine) causes in *Daphnia magna* intensification and prolongation of the effects of acetylcholine. Likewise, after eserinization of animals, the acetylcholine becomes effective on the intestine in a shorter period of time. Fifteen animals which were treated with physostigmine 1×10^{-4} for 2 minutes and then with acetylcholine 1×10^{-7} yielded results which are shown in Table IV. The reaction time for eserinized individuals in the production of vigorous muscular contrac-

tions when treated with acetylcholine 1×10^{-7} is shown to be on the average 50.1 seconds as compared with 104.4 minutes when acetylcholine of the same concentration is employed alone (see Table I).

The intestine of *Daphnia magna* responds to a treatment of physostigmine when employed alone in the same way as it does to acetylcholine. When 15 animals were treated with physostigmine 1×10^{-4}, vigorous intestinal contractions appeared in about 10 minutes (Table IV, column 2). This relatively strong concentration of physostigmine was employed because the utilization of this strength revealed certain manifestations in the course of the action of the chemical substance which were not observed when higher dilutions were employed. The animal under the influence of the drug becomes immediately immobile. The wall of the intestine becomes opaque due to an extreme contraction of the muscular fibers and the intestine enters into a state of contracture. After 2 or 3 minutes the organism gradually begins to recover its normal swimming movements and the intestinal wall commences to reëstablish its normal state. In time there appear extremely powerful intestinal contractions. These contractions with lapsed time become more intensified and persist for a considerably longer period than when acetylcholine alone is administered to the animals. This period was often observed to extend over one hour after the drug is replaced by water.

Discussion

The action of acetylcholine, atropine and physostigmine on the intestine of *Daphnia magna* is such that it strongly suggests the possibility that this organ is controlled by cholinergic nerves. Acetylcholine, when applied in the concentrations employed in this work, was shown to intensify the intestinal activity. This action of acetylcholine was shown to be antagonized by atropine and augmented and prolonged by physostigmine. These and other observations recorded in this paper are in accord with the rôle which has been ascribed to these substances in physiological processes where nervous impulses are involved and where acetylcholine is believed to act as a transmitter of nervous impulses.

The sudden appearance of vigorous muscular contractions of the intestine under the influence of acetylcholine have enabled us to obtain certain data pertaining to the time which elapses between the application of the chemical substance and the onset of the specific effect produced. It is of considerable interest and importance to note that whereas acetylcholine in concentrations of 1×10^{-2} and 1×10^{-3} produces vigorous intestinal contractions in less than 30 seconds, with further dilution of the drug this period is considerably prolonged before the accelerating

response of the intestine to acetylcholine is noted. With acetylcholine 1×10^{-4} the period becomes, on the average, 10.7 minutes and with acetylcholine 1×10^{-7} the time which elapsed between the addition of the chemical substance and the appearance of the characteristic response was shown to be on the average 104.4 minutes. Latent periods of such extreme magnitudes are not in accordance with our present knowledge pertaining to the action of chemical substances which are thought to act as chemical transmitters of nervous impulses.

In view of the observation recorded in this paper it may be assumed that the effectiveness of acetylcholine is dependent on the rate of penetration and diffusion of the drug to the site of action, and on the rate of destruction. Acetylcholine 1×10^{-7}, however, when preceded by physostigmine 1×10^{-4} produces vigorous intestinal contractions in *Daphnia magna* in less than one minute. This indicates that acetylcholine of this relatively weak concentration reaches the site of action quickly and that the rate of penetration and the rate of diffusion in this particular instance are not primarily factors. However, it is possible that the rapid destruction of the acetylcholine when unprotected by physostigmine is responsible for the long delays preceding the onset of its characteristic action.

Artemov and Mitropolitanskaja (1938) have demonstrated the presence in whole *Daphnia* of an acetylcholine-like substance. As yet, however, no one has undertaken to demonstrate the presence or absence of choline esterase in this group of animals. The questions of how acetylcholine, if present in *Daphnia magna,* is bound in the tissues and how it is protected must wait further investigations before they are answered.

Summary

1. Acetylcholine produces in *Daphnia magna* vigorous intestinal contractions which persist for some time after they are established.

2. The period which elapses between the addition of the acetylcholine and the onset of the characteristic effect is definitely dependent on the concentration of the drug employed.

3. Atropine blocks the action of acetylcholine.

4. Physostigmine causes intensification and prolongation of the effects of acetylcholine.

5. Acetylcholine, when it is preceded by physostigmine, causes in *Daphnia magna* a considerable reduction in the time which elapses between the administration of the drug and the appearance of the vigorous intestinal contractions.

LITERATURE CITED

Artemov, N. M., and R. L. Mitropolitanskaja, 1938. Content of acetylcholine-like substances in the nerve tissue and of choline esterase in the hemolymph of crustaceans. *Bull. de Biol. ed de Méd. Expér. U. R. S. S.*, **5**: 378–381.

Banta, A. M., 1921. A convenient culture medium for daphnids. *Science, N.S.*, **53**: 557–558.

Obreshkove, V., 1930. Oxygen consumption in the developmental stages of a cladoceran. *Physiol. Zoöl.*, **3**: 271–282.

Welsh, J. H., 1939*a*. Chemical mediation in crustaceans. I. The occurrence of acetylcholine in nervous tissues and its action on the decapod heart. *Jour. Exper. Biol.*, **16**: 198–219.

Welsh, J. H., 1939*b*. Chemical mediation in crustaceans. II. The action of acetylcholine and adrenalin on the isolated heart of Panulirus argus. *Physiol. Zoöl.*, **12**: 231–237.

Welsh, J. H., and H. H. Haskin, 1939. Chemical mediation in crustaceans. III. Acetylcholine and autotomy in Petrolisthes armatus (Gibbes). *Biol. Bull.*, **76**: 405–415.